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Award Number: W81XWH-05-1-0367

TITLE: Systemic Oncolytic Cytokine HSV Therapy of Prostate Cancer

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REPORT DATE: May 2007

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
01-05-2007	Annual	15 Apr 2006 – 14 Apr 2007

4. TITLE AND SUBTITLE

Systemic Oncolytic Cytokine HSV Therapy of Prostate Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-05-1-0367

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

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5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Boston, MA 02114-2554

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSOR/MONITOR'S ACRONYM(S)

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES

14. ABSTRACT

The purpose of the present work is to evaluate the use of HSV oncolytic vectors and their cytokine-containing derivatives as potential therapeutic modalities in treating prostate cancer. To this end, we have exploited the use of the transgenic TRAMP mouse, which recapitulates the developmental hallmarks of prostate cancer in humans. In last year's progress report, we made substantial strides in addressing all three specific aims. This year, we have added additional experiments to solidify this work and have subsequently submitted a manuscript entitled, "Systemic therapy of spontaneous prostate cancer in transgenic mice with oncolytic herpes simplex viruses" to Cancer Research to be reviewed for publication (see attached manuscript in appendix). Furthermore, we have recently implemented a novel complementary approach using human prostate explants derived from cancer biopsies to further assess the efficacy of oncolytic HSV virotherapy. Our preliminary data shows that oncolytic HSV's specifically targets epithelial cells within the prostatic ducts and remarkably, leaves the surrounding stroma unaffected.

15. SUBJECT TERMS
Prostate cancer, Oncolytic HSV

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
U	U	U	UU	40	19b. TELEPHONE NUMBER *(include area code)*

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Systemic Oncolytic Cytokine HSV Therapy of Prostate Cancer

Progress report August 2007 Brent J. Passer (PI)

INTRODUCTION

Oncolytic HSV-based vectors selectively replicate in tumor cells causing direct killing ie., oncolysis, while at the same time sparing normal cells. To better assess the utility of oncolytic HSV vectors in treating prostate cancer, we have taken advantage of a transgenic mouse model system called TRAMP, which develops prostate cancer spontaneously and closely mirrors the progression of prostate cancer seen in humans. In last year's progress report, we included data that addressed aspects from all three specific aims. Over the course of this year's work, we have extended and refined these studies and have subsequently submitted a manuscript entitled, "Systemic therapy of spontaneous prostate cancer in transgenic mice with oncolytic herpes simplex viruses" to *Cancer Research* to be reviewed for publication (see attached manuscript in appendices).

BODY

In last years progress report we demonstrated that:

1. Inbred TRAMP mice on a FVB/N background displayed a consistent and predictable temporal pattern of prostate cancer progression, ie., low grade PIN (8 weeks) high grade PIN (10 weeks), prostate adenocarcinoma (12 weeks) and metastasis to the lungs (18 weeks). (Aim 1)
2. Systemic delivery of oncolytic NV1042 (expressing IL-12) replicates within prostate tumors as defined by β-gal staining and quantitative PCR of HSV gB sequence. (Aim 2)
3. Oncolytic NV1023 and to a greater extent NV1042 vectors delivered systemically at 12 weeks of age promoted robust regression of prostate tumor growth by 24 weeks of age. (Aim 2)
4. NV1042 also diminished prostate tumor size even when delivered by 18 weeks of age (period of metastasis). (Aim 3)

In addition, to the above aforementioned results, we have also added additional data to solidify these results by:

1. Demonstrating a significant reduction in the frequency of metastasis to the peri-aortic lymph nodes after administration of either NV1023 or NV1042 (Mock 86% as compared to NV1023 (14%) and NV1042 (25%). (See Table 1; Aim 3).
2. Demonstrating a tendency toward a reduction of metastasis to the lungs by NV1042 and NV1023 treatments. Although this was not statistically significant. (See Table 1; Aim 3).
3. Showing that NV1042 persistence within the tumor as defined by β-gal staining is different from senescence-associated β-gal activity, which can arise in prostate hyperplasia. (See Figure 4B; Aim 2).
4. Verifying by immunohistochemistry that anti-β-gal staining mirrored X-gal staining. (See Figure 4A, g, h; Aim 2).

As NV0142 was effective at reducing tumor burden, we hypothesized that NV1042 in combination with NV1034, which expresses GM-CSF may act "synergistically" in order to promote enhanced tumor regression (See Aim 2). This rationale is based on our previous published observations that the administration of NV1042/NV1034 combination in a subcutaneous implantable prostate cancer model was more effective than either one alone (1). Thus, experiments were performed in which 10 week old TRAMP mice (N=8/group) received 4 injections spaced 3 days apart with either mock-treated PBS control, NV1042 ($2x10^7$ pfu), NV0134 ($2x10^7$ pfu) or the combination of NV0142 + NV1034 ($1x10^7$ pfu of each). Mice were allowed to develop until the age of 24 weeks, at which time tumors were harvested and analyzed. To our unfortunate surprise, we observed that tumors derived from all mock-treated animals had substantially smaller than normal-sized tumors. We subsequently examined over 40 additional TRAMP male mice at various stages of tumor development, which had been planned for additional experiments and confirmed that these mice also lost the ability to spontaneously generate prostate tumors. In addition, Dr. Petur Nielsen, a MGH pathologist and our collaborator, verified our initial assessment by inspection of H&E tissue sections on numerous tumor samples.

Recently, we have initiated the second of two breeding sequences required to obtain male TRAMP mice on a FVB/N background. That is, female transgenic F1 mice are currently being crossed with male FVB/N males. Once these litters are obtained, they will be genotyped using tail genomic DNA, weaned and prostate tumor progression will be initially re-evaluated before we commence with the aforementioned experiments.

In addition, we have developed a novel complementary approach towards assessing the effectiveness of oncolytic HSV therapy in prostate cancer (not in the grant application but highly relevant). This experimental paradigm takes advantage of previously published work in which human prostate cancer biopsies can be easily maintained *in vitro* on a collagen sponge for 1-2 weeks (2). The advantage of a prostate organ culture system is that the three-dimensional structure remains intact. Thus, all of the factors that may affect viral entry and replication, such as cell-cell, cell-matrix interactions, and interstitial fluid within this three-dimensional milieu remain preserved. An critical question that has never been addressed is whether oncolytic HSV vectors can efficiently and specifically target the epithelial cells within the prostatic ducts, which is the site of prostate cancer progression. To this end, human prostate cancer specimens were cut into 2-4 mm^3 fragments, pre-incubated for one hour with the oncolytic G47Δ virus ($1x10^6$ pfu), which is similar to NV1023 except that both copies of *γ34.5* genes have been deleted and the *α47* gene is absent, and thereafter, tumor fragments placed on a semi-submersed collagen sponge (Figure 1).

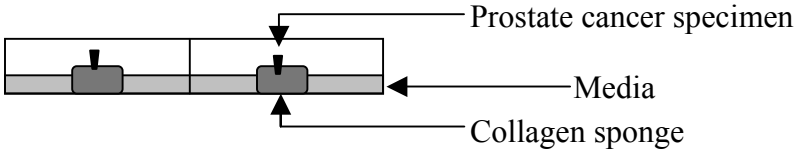

Figure 1. Prostate organ culture. Side-view of a 6-well plate containing prostate tumor fragments placed on a collagen sponge after being incubated with oncolytic HSV.

At days 2, 5 and 7 post-infection, prostate cancer tissues were fixed, sectioned and stained with either X-gal, to detect the presence of the virus (*ICP6* gene has been replaced by *LacZ*) or immunostained with anti-cytokeratin 18, which demarcates the epithelial cells within the prostatic ducts. Figure 2 shows that the G47Δ specifically targets the prostatic ducts. Strikingly, by D7, these ducts appeared extensively damaged presumably due to the oncolytic activity of G47Δ. It should be noted that in all cases examined, negligible X-gal staining was observed in uninfected prostate cancer specimens (data not shown). Furthermore, the X-gal staining pattern seen in Figure 2, mirrors the immunostaining pattern observed using anti-HSV-1 antibodies (data not shown). Thus, these data demonstrate that oncolytic G47Δ vectors can specifically target the epithelium of the prostatic ducts, but spares the surrounding stroma. Future experiments will focus on comparing these results with that of wild-type HSV (strain F) in order to examine differences in cell tropism. In addition, as we have shown that the murine IL-12 expressing NV0142 is effective in diminishing tumor burden in TRAMP mice, it is our goal to construct a G47Δ-human IL-12 vector not only to evaluate its lytic activity, but also test its robustness towards eliciting a T-cell mediated immune response within the prostate cancer microenvironment.

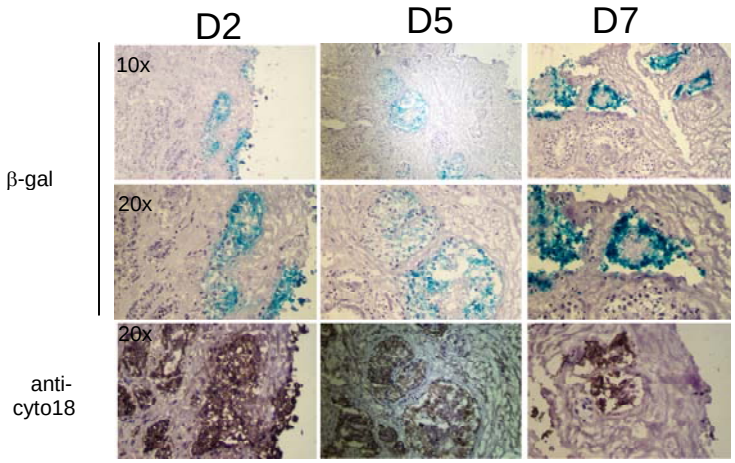

Figure 2. Oncolytic G47Δ specifically targets the prostatic ducts of human prostate cancer biopsies. Prostate cancer specimens were treated with G47Δ and day 2, 5 or 7 post-infection tissues were fixed and sectioned. Tissues sections stained with X-gal (blue) were photographed either at 10x (*upper row*) or 20x magnification (*middle row*). In addition, serial sections were immunostained with anti-cytokeratin18 (brown) (*bottom row*,) to demarcate the prostate epithelial cells within the ducts and to demonstrate that G47Δ infection overlaps with cytokeratin 18-expressing cells. Tissues sections were also counterstained with hematoxylin. Note the cauterized-like appearance of the prostatic ducts at D7 and the lack of B-gal^{+} cells in the surrounding stroma. Similar findings have also been obtained with NV1023.

KEY RESEARCH ACCOMPLISHMENTS

- Addressed key questions from all three specific aims.
- Added additional data to further validate last year's work.
- Submitted a manuscript to *Cancer Research*.
- Reported on a novel complementary approach towards addressing the effectiveness and specificity of oncolytic HSV vectors in prostate cancer.

REPORTABLE OUTCOMES

None

CONCLUSIONS

The use of the genetically engineered TRAMP mice and more recently, human prostate organ cultures derived from cancer specimens has allowed us to further validate the effectiveness of oncolytic HSV vectors as potential therapeutic modalities for the treatment of prostate cancer. It is our goal that by exploiting such experimental paradigms of prostate cancer, it will not only allow us to better understand the mechanisms by which viral oncolysis promotes tumor killing, but also aid in the further development of novel HSV-based vectors as well as in developing rationalized-based dual-therapy approaches, a commonly used regime for treating prostate cancer.

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APPENDICES

- **Manuscript:** Varghese et al, "Systemic therapy of spontaneous prostate cancer in transgenic mice with oncolytic herpes simplex viruses."
- **Figure 1.** Spontaneous primary and metastatic prostate cancer development in TRAMP mice.
- **Figure 2**. Efficacy of systemic oncolytic virus in TRAMP mice treated at 12 weeks of age.
- **Figure 3**. Efficacy of systemic oncolytic virus in TRAMP mice treated at 18 weeks of age.
- **Figure 4**. Biodistribution of NV1042 virus following intravenous injection of 12-week old TRAMP mice.

- **Figure 5**. Real time PCR for HSV gB sequences in various tissues from TRAMP mice treated at 18 weeks with oncolytic HSVs.
- **Table 1**. Frequency of prostate lesions and metastatic cancer in TRAMP mice treated with oncolytic HSVs.

SYSTEMIC THERAPY OF SPONTANEOUS PROSTATE CANCER IN TRANSGENIC MICE WITH ONCOLYTIC HERPES SIMPLEX VIRUSES

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Financial support: This research was supported by grants to RLM from NIH (1R01CA102139-01A1), Department of Defense (DAMD17-98-1-8490, W81XWH-04-1-0254), and to SV from Department of Defense (W81XWH-05-1-0367). The real-time PCR core was supported by a grant from NIH (P30 NS045776).

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Running title: Systemic Oncolytic HSV Therapy Of Spontaneous Prostate Cancer

Key words: Oncolytic HSV, Systemic Therapy, Spontaneous Prostate Cancer, TRAMP, Transgenic Mice

ABSTRACT

Oncolytic viruses are an innovative therapeutic strategy for cancer, where viral replication and cytotoxicity are selective for tumor cells. Here we demonstrate the efficacy of systemically administered oncolytic viruses for the treatment of spontaneously arising tumors. Specifically, the use of oncolytic herpes simplex viruses (HSVs) administered intravenously to treat spontaneously developing primary and metastatic prostate cancer in the transgenic TRAMP mouse, which recapitulates human prostate cancer progression. Four administrations of systemically delivered NV1023 virus, an HSV1/2 oncolytic recombinant, to TRAMP mice at 12 or 18 weeks of age (presence of prostate adenocarcinoma or metastatic disease, respectively) inhibited primary tumor growth and metastases to lymph nodes. Expression of IL-12 from NV1042 virus, a derivative of NV1023, was additionally effective, significantly reducing the frequency of prostate carcinoma and lung metastases, even when the mice were treated after the onset of metastasis at 18 weeks of age. NV1042-infected cells, as detected by X-gal staining for *Lac Z* expressed by the virus, were present in prostate tumors 1 week after the final virus injection and viral DNA was detected by real time PCR in primary and metastatic tumors, but not in liver or blood, at 2 weeks after final virus injection. No toxicity was observed in any of the treated mice. The efficacy of the IL-12 expressing NV1042 virus in this aggressive prostate cancer model using a clinically relevant treatment paradigm merits its consideration for clinical studies.

INTRODUCTION

Oncolytic viruses designed to differentially target cancer cells while sparing normal tissues have advanced in the past decade to the forefront of innovative strategies for cancer treatment (1, 2). Since the conception in 1991 of using mutated *herpes simplex* virus (HSV), a neurotropic virus, to treat brain tumors (3), the unique biology of HSV coupled with genetic manipulation techniques have greatly aided in the development of more potent HSV vectors while conferring safety and specificity (4). Currently, four oncolytic HSV vectors, G207, HSV 1716, NV1020, and Oncovex$^{GM\text{-}CSF}$, delivered intracerebrally, intraneoplastically or intra-arterially have successfully completed phase I clinical trials (5, 6). These and other oncolytic HSVs have been efficacious in treating a variety of cancers in animal models (4, 7). In addition to their direct tumoricidal effect, oncolytic herpes viruses are also capable of eliciting an anti-tumor immune response (8-10), an important feature when treating metastatic tumors, especially those that are clinically occult.

Accumulating evidence suggests that oncolytic HSV mutants are potentially useful agents for the treatment of prostate cancer: (i) G207, a multi-mutated HSV-1 (11), is safe when administered into the prostate in pre-clinical animal models, and is a nerve-sparing virus (12, 13). This overcomes a current challenge of conventional treatments, such as, surgery and radiation therapy, which are associated with risks of nerve damage resulting in urinary incontinence, proctitis, and erectile dysfunction. (ii) A variety of oncolytic HSV mutants, including G207 and NV1020 have demonstrated efficacy against subcutaneous human prostate cancer xenografts and mouse prostate cancers following intraneoplastic or intravenous administration (14-18). (iii) G207 and other vectors are effective against human prostate cancer irrespective of hormone

status or radio-sensitivity (14, 16, 19)- a major advantage in its application for advanced forms of the disease, where hormone and radiation refractory tumor is an inevitable progression.

To-date, however, all efficacy studies with HSV vectors for prostate cancer has utilized implanted tumor models, which are artificial systems with respect to their milieu and lympho-vascular supply. While these implanted models are easily amenable to therapeutic manipulation, they do not truly reflect the *in situ* cancer situation and may affect the outcome of the therapy being investigated. Genetically engineered mouse models which develop prostate cancer spontaneously are currently the most representative models to conduct efficacy studies. Therefore, we have utilized the transgenic TRAMP mouse, which develops prostate cancer spontaneously with progression to metastatic disease (20, 21). In TRAMP mice, the rat probasin promoter regulated by androgens drives SV40 T antigen, thus restricting its expression to epithelial cells of the prostate. Histological progression of prostate cancer in TRAMP mice closely recapitulates that of humans, with the development of prostatic intraepithelial neoplasia (PIN) by 8 weeks of age, prostate carcinoma by 12 weeks, and metastatic cancer in peri-aortic lymph node (PA-LN) and lungs by 18 weeks of age (21).

As a prelude to the studies in TRAMP mice, we examined the efficacy of various oncolytic HSVs (G207, NV1023, and NV1042) for subcutaneous and metastatic lung tumors using a prostate cancer cell line (TRAMP-C2), which was established by Greenberg and colleagues from a spontaneously occurring prostate adenocarcinoma obtained from a TRAMP/C57Bl6 mouse (20). NV1023 is derived from NV1020, which has been in clinical trial for metastatic colon cancer to the liver, and has deletions of one copy of γ34.5, the internal repeat, and UL24 and

UL56 genes, as well as the addition of gJ, gG, US2 and US3 genes from HSV-2 (22, 23). NV1023 has an additional insertion of LacZ and deletion of ICP47, US11 and US10 genes (22). Comparison of efficacy in subcutaneous or metastatic lung TRAMP-C2 tumors demonstrate that the mIL-12 expressing NV1042 virus was more superior than its parental virus, NV1023, or G207 (17, 18). We also demonstrated that IL-12 expression from NV1042 resulted in both immune and anti-angiogenic effects (17, 18).

Based on the above findings, we have further utilized the IL-12 vector, NV1042, in this current study to investigate systemically administered NV1023 and NV1042 to treat spontaneously arising prostate cancer and metastasis in TRAMP mice. The results demonstrate that both NV1023 and NV1042 significantly inhibited the growth of primary tumors in the prostate and metastasis in the peri-aortic lymph nodes. NV1042 was additionally effective in reducing the frequency of prostate carcinoma and lung metastasis.

MATERIALS & METHODS

Mice: TRAMP (C57Bl/6 background) breeder pairs (female TRAMP and male C57Bl/6) were purchased from The Jackson Laboratory (Bar Harbor, Maine) and bred in-house at the Center for Comparative Medicine facility at Massachusetts General Hospital (MGH). Female transgenic F1 pups were crossed with male FVB/N mice obtained from NCI (Bethesda, MD) to generate TRAMP mice on an FVB/N background. The pups were genotyped at 3 weeks of age using SV40 large T antigen primers (5'-CAGAGCAGAATTGTGGAGTGG-3' and 5'-ACAAACCACAACT AGAATGCAGTG-3') for PCR of tail genomic DNA isolated using phenol chloroform extraction (24). F1 male TRAMP mice obtained from this cross- breeding

were used for all experiments described below. Mice were housed in a pathogen-free facility and all animal procedures were conducted with approval from MGH Subcommittee on Research Animal Care. All animal studies were blinded.

Viruses: Purified virus stocks of NV1023 and NV1042 were obtained from MediGene Inc (San Diego, CA). Construction of NV1023 and NV1042 has been described previously (22). NV1023, derived from NV1020 (R7020), a HSV-1/HSV-2 intertypic recombinant developed as a vaccine strain (23), contains an insertion of LacZ into the ICP47 locus, deleting ICP47, US11 and US10 (22). NV1042 is NV1023 with an insertion of murine IL-12 cDNA (p35 and p40 as a single polypeptide separated by elastin motifs) expressed from a hybrid α4-TK promoter (22). NV1042 infected TRAMP-C2 cells secreted 52 ng/ml of IL-12 (17). The viruses were individually titered on Vero (African green monkey kidney) cells by plaque assay.

Virus Treatment and Efficacy Evaluation: Twelve or 18 week old male TRAMP mice were inoculated via tail vein with $2x10^7$ plaque forming units (pfu) of NV1023 or NV1042 or virus buffer consisting of 10% glycerol in phosphate buffered saline (Mock) in a 200 µl volume, on days 0, 3, 7, and 10. By day 14 after initiation of treatment, anti-HSV serum antibody was detectable (data not shown). Mice were monitored biweekly and sacrificed if morbid. At 24 weeks all mice were sacrificed terminating the experiment. Prostate and seminal vesicles were removed *en bloc*, weighed and photographed. Formalin fixed sections of prostate, peri-aortic lymph nodes and lungs were evaluated by H&E staining and the frequency of carcinoma was scored in a blinded manner by the collaborating pathologist (G.P.N).

Virus Bio-distribution Studies: TRAMP mice were treated with $2x10^7$ pfu of NV1042 on days 0, 3, 7, and 10. Mice were sacrificed at pre-determined days and various tissues were evaluated for *ß-galactosidase* by X-gal staining/immunohistochemistry and by real time PCR for presence of HSV-1 DNA.

ß-galactosidase Staining: Tissue cryostat sections of prostate/ seminal vesicles, peri-aortic lymph nodes, lung, liver, and brain obtained from 3 mice each sacrificed on days 11, 13, and 17 (or 1, 3, and 7 days after the final treatment) were analyzed by X-gal staining at pH 7.2, as described previously (17). For senescence-associated (SA) ß-galactosidase staining, we performed X-gal histochemistry at pH 6.0 (25). Sections were counter-stained with eosin or hematoxylin & eosin. For immunohistochemistry, sections were washed with 0.2% Triton X-100 in PBS, 0.3% hydrogen peroxide in PBS, 1% and then 10% goat serum in PBS, incubated with rabbit anti-*E.coli* ß-galactosidase (1:1000; Abcam Inc, Cambridge MA) overnight at 4ºC, washed in PBS and incubated with biotinylated goat anti-rabbit IgG (Vector Laboratories, Burlingame, CA). Immunoreactive material was detected with Vectastain Elite ABC and diaminobenzidine kits (Vector Laboratories).

Real time PCR: NV1042 treated mice were sacrificed on day 11, 13, and 24 (or 1, 3, and 14 days after the final treatment) and various tissues (prostate/ seminal vesicles, peri-aortic lymph nodes , lung, liver, brain, and blood) were removed aseptically and immediately snap frozen in dry-ice with isopentane. Tissues were resuspended in nucleic acid lysis buffer (Applied Biosystems, Foster City, CA) and homogenized using a Mixer Mill (Qiagen, Carlsbad, CA). Total DNA was extracted from the homogenate using ABI Prism 6100 Nucleic Acid PrepStation (Applied Biosystems). Absolute quantification of viral DNA was conducted by real time Taqman PCR using HSV-1 gB primer sequences (Forward primer: 5'-TGTGTACAT GTCCCCGTTTTACG-

3’; Reverse primer: 5’-GCGTAGAAGCCGTCAACCT-3’; Probe: 5’-ACACCAG CTACGCCGCC-3’) synthesized using assays-by-design service (Applied Biosystems). Mouse GAPDH primers (Applied Biosystems) were used as endogenous control for input DNA. Strain F genomic DNA served as positive control and was used to generate a standard curve from 15 to $2.4x10^5$ copies.

Statistical Analysis: Since the experimental data of prostate tumor weight from the efficacy studies did not follow a normal Gaussian distribution, non-parametric Mann-Whitney tests (two-tailed) were used to analyze significance between 2 treatment groups. The frequency of carcinoma in prostate, PA-LN and lungs between 2 treatment groups were conducted by contingency analysis using Fisher’s exact two-sided test. Kaplan Meier survival data were analyzed using chi square Log rank test. Alpha levels for all analyses were $P<0.05$; n values and exact *P* values are indicated in the text and legends. All statistical analyses were done using GraphPad prism v.4 (San Diego, CA).

RESULTS

Spontaneous primary and metastatic prostate cancer development: Since the TRAMP mice were bred in-house, we determined the time line of prostate cancer and metastasis development prior to using them in viral therapy studies. As illustrated in Figure 1, TRAMP mice on the FVB/N background display low grade PIN by 8 weeks of age (Fig 1B), which progresses to high grade PIN (adenoma) by 10 weeks (Fig 1C), prostate adenocarcinoma by 12 weeks (Fig 1 D), and metastasis in peri-aortic lymph nodes and lung by 18 weeks of age (Fig 1 E,F). For comparison, histology of normal prostate from a non-transgenic littermate is shown in Figure

1A. Systemic treatment with NV1023 or NV1042 was initiated at an age when mice first exhibit either primary prostate carcinoma (12 weeks) or metastasis (18 weeks), and the mice were sacrificed at 24 weeks of age, an age where untreated mice become moribund from disease. A mock mouse sacrificed at 24 weeks of age (illustrated in Figure 1G) displays a primary prostate tumor and metastasis in peri-aortic lymph nodes, lung, and liver. Interestingly, although the large size of the prostate cancer and metastatic lymph nodes is representative of the highly aggressive nature of prostate cancer in the TRAMP mice, this was the only mouse where overt metastasis in the liver was observed.

Efficacy of systemic oncolytic HSV therapy on primary prostate cancer: Mouse cells are more resistant to HSV infection and in our prior studies with implanted mouse prostate TRAMP-C2 cells in C57Bl/6 mice, we had noted that 4 intraneoplastic injections were significantly more effective than 2 treatments (17). Additionally, for treating TRAMP-C2 tumors metastatic to lung, we had observed that 4 intravenous administrations were significantly effective in inhibiting the growth of the tumors. Therefore, for testing in this spontaneous model, 12 week-old TRAMP mice were treated intravenously with four doses of $2x10^7$ pfu of NV1023 or NV1042 which resulted in substantial inhibition of primary prostate cancer growth when compared to Mock mice as illustrated in the gross photograph (Fig. 2A). Since multifocal tumors also arise in the seminal vesicles of these mice and often coalesce with the prostate gland, the carcinomatous mass containing both prostate and seminal vesicles was excised as one unit and weighed at the time of sacrifice (24 weeks). Distribution of prostate/seminal vesicle tumor weights (Fig. 2B) illustrates that Mock-treated mice harbored tumors with a mean weight of 10.17g, NV1023 with 3.98g (P=0.026 vs. Mock; Mann-Whitney test) and NV1042 with 2.79g

(*P*=0.003 vs. Mock; Mann-Whitney test). For comparative purposes, the average weight of prostate and seminal vesicles from non-transgenic TRAMP mice is 0.78 g. In this experiment, 2 of 9 mice from both the Mock and NV1023 treated groups died within 2 days of the 24 week sacrifice and 1 mouse from the NV1042 group died one week after treatment (at ~14 weeks of age). Histological analysis of prostates from these dead mice showed that those from the Mock and NV1023 treatment groups had large prostate tumors comprising of carcinoma, whereas the single NV1042 treated mouse did not display any evidence of cancer and therefore likely died from unrelated causes. H&E analysis of prostates confirmed the presence of poorly differentiated carcinoma in 8 out of 9 (89%) Mock mice as compared to 6 out of 9 (67%) in NV1023 and 2 out of 8 (25%) in NV1042 treated mice (*P*=0.015 vs. Mock, Fisher's Exact test). Representative H& E stained prostates, based on the most frequently observed histological stage, from various treatment groups are illustrated in Fig 2C-E. The largest tumors were highly necrotic (seen as pink areas in Fig 2C) with islands of tumor cells closely apposed to blood vessels within the necrotic areas.

Both NV1023 and NV1042 also inhibited primary prostate tumor growth as compared to Mock treatment in mice treated at 18 weeks of age, when they begin to exhibit metastasis (Fig. 3A). There was a significant difference in the number of animals surviving to 24 weeks, with 8 out of 17 Mock dying or being sacrificed between 22-24 weeks due to tumor burden, as compared to 3 of 17 NV1023 and 2 of 17 NV1042 treated (p = 0.03; chi square log rank test). All of these mice harbored carcinoma within the prostate as determined by histological analysis. Comparison of prostate/seminal vesicles weights in mice sacrificed at 24 weeks (Fig. 3B) demonstrates a mean weight of 12.25g in Mock versus 6.54g in NV1023 (*P*=0.04 vs. Mock; Mann-Whitney test) and

3.66g in NV1042 treated mice (*P*=0.002 vs. Mock, Mann-Whitney test). Histological analysis revealed that 16 out of 17 (94%) Mock mice, 13 out of 17 (76%) of NV1023, and 10 out of 17 (59%) of NV1042 treated mice (*P*=0.039 vs. Mock, Fisher's exact test) harbored prostate adenocarcinoma. Thus, while both NV1023 and NV1042 were equally effective in inhibiting the growth of primary tumors when treated at 12 or 18 weeks, only NV1042 was effective against progression to carcinoma within the prostate.

Efficacy of systemic oncolytic HSV therapy on metastasis: Treatment of TRAMP mice with NV1023 or NV1042 at 12 weeks of age, when they begin to develop prostate carcinoma, resulted in a significant reduction of metastatic frequency in peri-aortic lymph nodes from 86% in Mock to 14% in NV1023 treated mice (*P*=0.03 vs. Mock, Fisher's Exact test) and to 25% in NV1042 (*P*=0.04 vs. Mock, Fisher's Exact test). While there was a reduction in metastatic frequency in the lungs, it was not significant (Table 1).

In order to examine whether oncolytic viral therapy could treat metastasis after their onset, TRAMP mice were treated at 18 weeks of age and sacrificed 6 weeks later at 24 weeks (Table 1). A significant reduction in frequency of metastasis to the peri-aortic lymph nodes was observed with NV1023 (*P*=0.04 vs. Mock, Fisher's Exact test) and NV1042 (*P*=0.013 vs. Mock, Fisher's Exact test), similar to the observations for treatment at 12 weeks of age. Interestingly, when scored for lung metastasis, a significant reduction was observed in the NV1042 treated mice, with 1 out of 14 (7%) mice exhibiting carcinoma (*P*=0.014 vs. Mock, Fisher's Exact test) as compared to 5 out of 9 (56%) Mock and 5 out of 14 (36%) NV1023 treated mice.

Virus bio-distribution in NV1042 treated mice: Both NV1023 and NV1042 harbor the *E.coli* Lac Z gene, which acts as a reporter to track these viruses following systemic administration. TRAMP mice treated at 12 weeks with four doses of $2x10^7$ pfu of intravenous NV1042 were sacrificed 1, 3, and 7 days after the last treatment, and various tissues (prostate, lung, liver, brain) removed for X-gal staining. Results as illustrated in Figure 4A show that one day following the final virus injection X-gal staining was observed in a few isolated cells in the liver and lung and none in brain (brain data not shown). However, by day 3 and 7 no staining was observed in the lungs, liver, or brain, whereas in the prostate, significant amounts of staining were detected. Thus, systemically administered NV1042 was able to persist at least for 7 days post-treatment in the cancerous prostates but not in the normal organs. X-gal staining seen in hyperplastic glands on day 1 was distinct from senescence-associated (SA) ß-galactosidase (Figure 4B) that has been reported in prostate hyperplasia(26, 27). To further confirm that X-gal histochemistry was identifying LacZ expressing cells, immunohistochemistry positive cells were seen in the same region as X-gal staining cells (Figure 4A g, h).

Biodistribution of NV1042 in 18 week-old TRAMP mice treated systemically on days 0, 3, 7 and 10 was assessed by real-time PCR using HSV gB sequences. DNA was isolated from organs (prostate/seminal vesicles, peri-aortic lymph nodes, lung, liver, brain, and blood) harvested on days 11, 13, and 24. Results shown in Figure 5 illustrate that viral DNA was detected until day 24 in those organs that harbor primary and metastatic cancers (prostate, peri-aortic lymph nodes, and lungs). In contrast, while many viral copies were detected in the liver and blood on day 1, the level decreased to non-detectable (negative) by day 24, suggesting clearance of virally

infected cells or degradation of viral DNA. No viral DNA was detected in the brains of any animal on any day tested.

DISCUSSION

Prostate cancer in TRAMP mice arises from the targeted expression of SV40 T antigen within the epithelial cells of the prostate (20), and is therefore influenced by the local prostate milieu. Studies utilizing TRAMP mice would be expected to be superior to implanted tumor models for a number of reasons: (i) Unlike implanted tumors generated from cultured cells which are usually of a homogenous clonal phenotype, prostate tumors in TRAMP mice arise multifocally, and are therefore heterogeneous in nature, similar to the clinical situation. Such differences also suggest that it would be more difficult to treat and cure multiclonal autochthonous TRAMP tumors as opposed to the implanted tumors, requiring more robust treatment regimens. (ii) Cells grown *in vitro* may accumulate additional alterations distinct from the original tumor, thus potentially influencing the outcome of therapies tested and affecting clinical translation. In contrast, evaluation of autochthonous tumors arising *in situ* would minimize such external influences. (iii) *In situ* prostate cancer development as observed in TRAMP mice is a dynamic process between transformed cells and the surrounding stroma and vasculature (28-30). In contrast, implanted tumors are in an artificial environment with respect to stroma, vasculature and lymphatic supply, and therefore, may respond to therapies more effectively, especially when initiated at a short interval after implantation when the tumor and local stromal cells have not become responsive to one another.

The TRAMP mice used in this study were bred on a FVB/N background. We also attempted to breed the TRAMP mice obtained from Jackson Labs on a C57Bl/6 background, but none of the 250+ F1 mice advanced from prostate adenoma to carcinoma even at death, which varied from 40 to 52 weeks. This lack of carcinoma development in the C57Bl/6 background is at variance with the original report of TRAMP mice (21) but could be attributed to genetic polymorphisms (31), or dietary and environmental influences (32, 33). Nevertheless, when the F1 pups from the C57Bl/6 background were crossed with the FVB/N background, the F1 pups from this cross simulated the time line of prostate cancer progression reported previously (34). Even these TRAMP X FVB/N pups exhibited variations in cancer development that differed from previous reports: (i) A majority (~75%) of our mice survived only until 25 weeks as opposed to a previously reported range of 24-39 weeks (35); and (ii) none of 80 mice over 18 weeks of age displayed bone metastases (21), which may have been due to our inability to detect the occasional incidence of bone metastasis reported in these mice. We also observed some litter / cohort variation which included (i) the rate of penetrance of prostate carcinoma, with some cohorts displaying 100% penetrance while others showed less (90%), and (ii) the survival rate of mice to 24 weeks (as described in the results), where only 22% of mice in the 12-week old treatment experiment died, where as, 47% in the 18-week old treatment experiment died. Such variations highlight the difficulty in conducting treatment studies with spontaneous tumor models, and in fact, most of the literature utilizing TRAMP mice has focused on prevention as opposed to treatment studies (36).

The route of virus administration will affect the proportion of infected tumor cells and efficacy, with intraneoplastic injection being the most direct. However, we administered virus

systemically for several reasons. (i) Since the virus was most effective when administered four times based on our prior studies with TRAMP-C2 implanted tumors, indicating that multiple treatments with oncolytic HSV were necessary to significantly inhibit mouse tumors (17). However, repeated laparatomies for local virus delivery into the prostate would have greatly increased the risk of procedure-related toxicity; (ii) For metastatic cancer, systemic administration would be the only route of delivery likely to reach the various metastatic sites. (iii) From a translational perspective, intravenous administration is much more amenable than a surgical procedure. In this study, after intravenous injection, NV1042-infected cells were detected within the prostates harboring cancer, and viral DNA was detected in the cancerous prostates, peri-aortic lymph nodes, and lungs, suggesting that systemically administered virus was able to reach and persist in those organs harboring tumors but not in normal organs (liver and brain), which cannot support efficient replication of the virus. Multiple injections of virus did not appear to be toxic, and tumor progression accounted for observed morbidity.

Both NV1023 and NV1042 treatment of TRAMP mice resulted in a significant reduction of primary prostate tumor weight irrespective of the age (12 or 18 weeks) at which treatment was initiated, however, NV1042 was additionally effective in inhibiting the extent of carcinoma *in situ*. Those NV1042-treated mice that were carcinoma-free at sacrifice, only displaying pre-neoplastic changes from PIN to adenoma within the prostate could result from two possible scenarios. (i) In some mice, NV1042 virus destroyed all pre-existing cancer cells at treatment and possibly by virtue of its IL-12 expression, activated the immune system, thereby protecting them from future carcinogenesis. (ii) In others the PIN never progressed to carcinoma, for example 6-11% of the Mock treated group did not harbor carcinoma *in situ* at the time of

sacrifice (24 weeks). Both NV1023 and NV1042 significantly inhibited the frequency of metastasis in peri-aortic lymph nodes independent of the age (12 or 18 weeks) at which treatment was initiated. However, only NV1042 but not NV1023 was significantly effective against metastasis in the lung when treated after the onset of metastasis at 18 weeks of age.

NV1042 has previously been shown to be significantly better at inhibiting tumor growth in squamous cell, hepatic, and colorectal carcinomas (22, 37, 38). Augmented efficacy of IL-12 expression in other oncolytic HSV vectors has also been reported (39, 40). We have previously compared the efficacy of NV1042 to NV1023 in subcutaneous and lung metastatic models using implanted TRAMP-C2 tumor cells and observed varying results depending upon the model ((17) versus (18)). The microenvironment and immune response to subcutaneous versus lung tumors is different, with NV1042 much more efficacious (in terms of survival) than NV1023 in the metastatic lung tumor compared to the subcutaneous model, even though the subcutaneous tumors were directly injected. In a bilateral subcutaneous tumor model, NV1042 had only a minimal effect on non-inoculated tumor growth (17), whereas in the metastatic lung model the enhanced efficacy of NV1042 over NV1023 was abrogated in athymic mice (18).

While NV1042 was significantly more efficacious than Mock in the TRAMP mice in almost all of the outcome measures and NV1023 was only significantly better in less than half, there were no statistically significant differences observed between NV1042 and NV1023, as opposed to the TRAMP-C2 metastatic lung model. Possible reasons for this difference include: (i) The implanted TRAMP-C2 tumors are of clonal origin and represent only a subset of the heterogenous tumor masses that arise multifocally in the spontaneously arising prostate cancers.

Importantly, the spontaneously arising tumors are continually progressing to more malignant phenotypes with time (from PIN to carcinoma to metastases), along with associated changes in the microenvironment and immune phenotype. (ii) The spontaneous prostate tumors arising *in situ* within the prostate gland are enclosed within a well-defined capsule thus potentially limiting the diffusion of systemic virus and the IL-12 expressed from NV1042 virus. The lung is the efferent side of a tail vein injection, therefore, in the metastatic lung model viruses were delivered directly to the lung as compared to the longer more indirect route to the spontaneous prostate tumors. Such variations in response to the same virus depending on the type of tumor model highlights the importance of evaluating oncolytic viruses in more than one model while emphasizing the need to use models such as the spontaneous tumor bearing TRAMP mice that are most representative of *in situ* prostate cancer development and progression.

We believe this is the first report of treating a spontaneous cancer model using systemically administered oncolytic HSVs. We recently reported the intraneoplastic use of oncolytic HSVs in the C3(1)/T-Ag model, which develops mammary tumors spontaneously (41). Although a direct comparison of NV1023 and NV1042 was not conducted, NV1042 significantly delayed mammary tumor progression as compared to Mock. Data from both these studies substantiates the utility of NV1042 against spontaneous tumor models, whether administered intraneoplastically or intravenously. An IL-12 expressing vector may have some advantages over a non-cytokine vector: (i) IL-12 binds to receptors on T and NK cells, enhancing their proliferation and cytotoxicity, driving a T_H1 response, and inducing interferons, which mediate many of its pro-inflammatory activities. It is a central immunoregulator and acts as a cross-bridge between innate and adaptive immunity (42); therefore, when expressed at the site of

tumor antigen production it can boost both arms of the immune response. (ii) Direct administration of cytokines into the tumor without sufficient tumor antigens or ‘danger signals’ can preclude an effective antitumor immune response. In contrast, an IL-12 expressing oncolytic virus, which can cause both tumor destruction and deliver an immune-enhancing cytokine in the vicinity of tumor destruction, would be highly beneficial. (iii) IL-12 is also an anti-angiogenic agent (43, 44).

The current study of systemic HSV treatment in an aggressive spontaneously developing prostate cancer model advances the validity of using oncolytic HSV therapy for prostate cancer patients, especially those with metastatic disease, who are severely limited in their treatment options. Importantly, in our study, treatment was initiated not just after primary tumors had developed, but also after metastases were apparent, similar to the situation in clinical practice. Furthermore, human cancer cells (including prostate cancer) are more susceptible to HSV oncolysis than mouse cells ((14) vs. (17)). The TRAMP mouse model is therefore a stringent test for efficacy and it might be expected that the results noted in this animal model could indicate even further efficacy when tested in patients. We have demonstrated that systemically administered oncolytic HSVs, in particular the IL-12 expressing NV1042 virus, was effective against not only the primary tumor but also metastatic tumors independent of their location. These desirable therapeutic features of NV1042 render it a highly valuable agent either as a primary treatment option or as an adjuvant following surgery to eliminate micrometastases.

ACKNOWLEDGEMENTS

We thank Meredith Regan from Dana Farber/ Harvard Cancer Core for assistance with the statistical analyses, Melissa Marinelli and Wenzheng Wang with the breeding, Dana Xu and Thanh Thao Huynh with histology, and Luba Zagachin with real-time PCR. RLM and SDR are consultants to MediGene AG, which provided us with the viruses tested in this study.

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LEGENDS

Figure 1: Spontaneous primary and metastatic prostate cancer development in TRAMP mice. (A) A normal prostate gland from a non-transgenic male littermate; **(B-F)** Time line of prostate and metastatic cancer development in TRAMP mice starting at 8 weeks of age when they develop low grade PIN **(B)**, and progressing to high grade PIN by 10 weeks of age **(C)**. Prostate adenocarcinoma is observed as early as 12 weeks of age **(D),** and metastasis in PA-LN **(E),** and lungs **(F),** are observed by 18 weeks of age. **(G)** Internal organs of a Mock mouse sacrificed at 24 weeks of age displaying large primary prostate tumor and metastasis in PA-LNs, liver and lung.

Figure 2: Efficacy of systemic oncolytic virus in TRAMP mice treated at 12 weeks of age. (A) Photograph of representative prostate and seminal vesicles excised *en bloc* from various treatment groups illustrating prostate tumors. **(B)** Distribution of weights of prostate/seminal vesicle from Mock (n=7), NV1023 (n=7), and NV1042 (n=8). Mean is denoted by the line and mean weight ± SEM for each group are as follows: Mock = 10.17± 1.68g; NV1023 = 3.98± 1.39g (P = 0.026 vs. Mock; Mann-Whitney test); NV1042 = 2.79± 0.91g (P = 0.0037 vs. Mock; Mann-Whitney test). Mice dying prior to week 24 were not included in the analysis. **(C-E)** H&E staining of prostates from Mock **(C)** showing larger areas of necrosis, NV1023 **(D),** and NV1042 **(E)** treated mice.

Figure 3: Efficacy of systemic oncolytic virus in TRAMP mice treated at 18 weeks of age. (A) Photograph of representative prostate and seminal vesicles excised *en bloc* from various treatment groups illustrating prostate tumors. **(B)** Distribution of weights of prostate/seminal

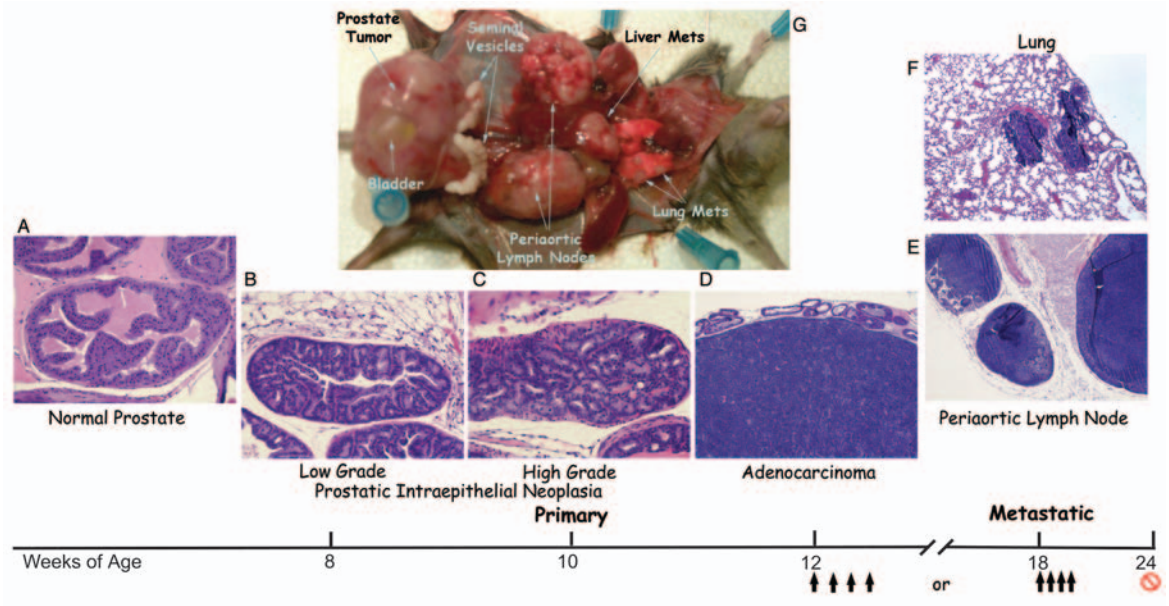
vesicle from each treatment group. Mean is denoted by the line in each group; mean weight ± SEM for each group are as follows: Mock = 12.25± 2.3g; NV1023 = 6.54± 1.20g (*P* = 0.04 vs. Mock; Mann-Whitney test); NV1023 = 3.66± 0.72g (*P* = 0.002 vs. Mock; Mann-Whitney test).

Figure 4: Biodistribution of NV1042 virus following intravenous injection of 12-week old TRAMP mice. A. Tissues harvested 1, 3 and 7 days after 4 viral injections were sectioned and stained with X-gal or anti-ß-galactosidase antibody to detect LacZ expression from the virus. **(a-c)** sections from prostate, liver and lungs obtained from mice sacrificed 1 day after treatments showing areas of staining in prostate glands with low grade PIN and a few X-gal positive cells in liver and lung (arrows). **(d-e)** sections from tissues 3 days post-virus injections showing X-gal staining in prostate but not in liver; **(f-h)** prostate tissue sections stained at 7 days post-virus injections. **f** and **g** are from different mice. The section in **h** was stained with anti-ß-galactosidase antibody. Note the overlapping LacZ immunohistochemical and X-gal histochemical staining in **h** and **g** (open arrowheads), which are from nearby sections. **B.** Senescence-associated β-galactosidase staining in Mock-treated prostate tumors. Frozen prostate sections from a mock-injected TRAMP mouse at 12 weeks (as in A) were histochemically stained at the same time for SA-β-Gal (left panels) and X-gal (right panels). Cells staining blue are positive. SA-β-Gal was not detected in high grade PIN or adenocarcinoma. In one mock-treated mouse, small clusters of positive-staining cells were seen in prostate tumors after X-gal histochemistry, likely due to endogenous activity (45).

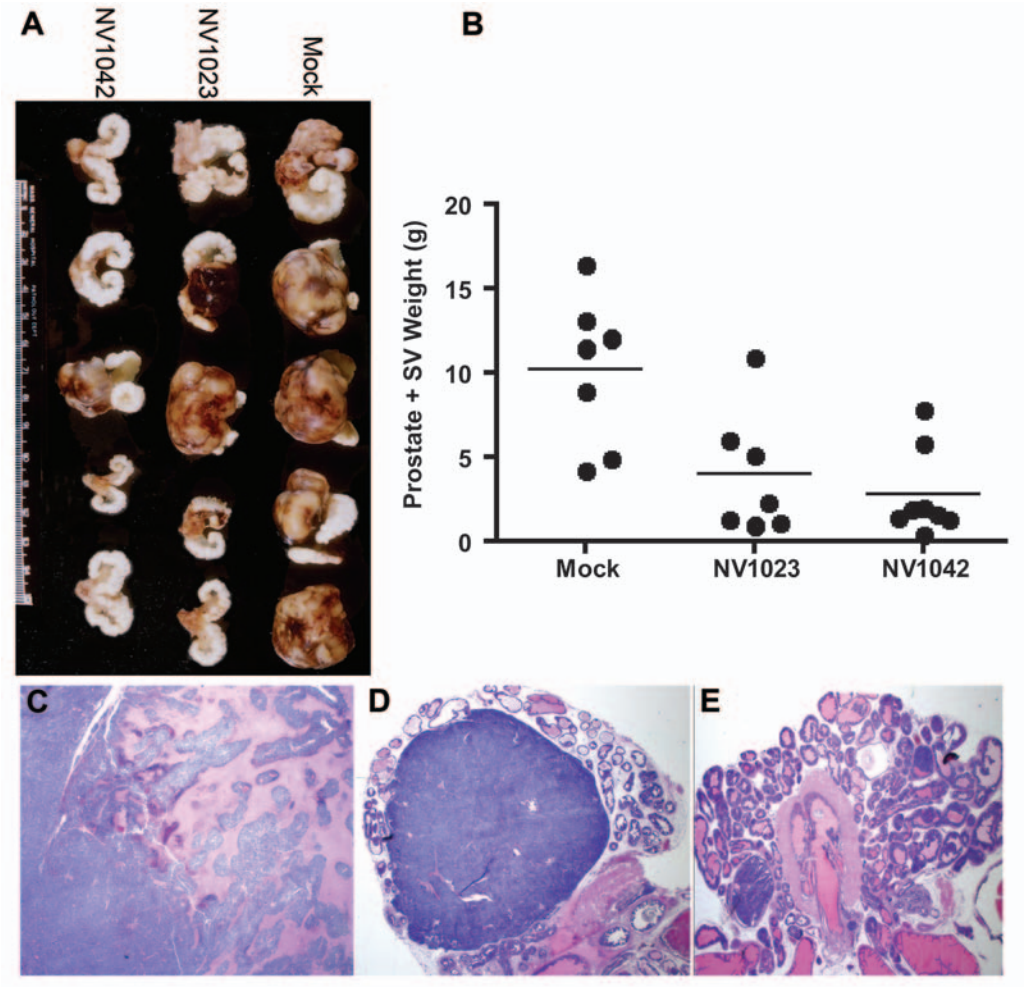
Figure 5: : Real time PCR for HSV gB sequences in various tissues from TRAMP mice treated at 18 weeks with oncolytic HSVs. NV1042 ($2x10^7$ pfu) was administered systemically

on days 0, 3, 7, and 10 in TRAMP mice at 18 weeks of age and various tissues were harvested on day 11, 13, and 24 for absolute quantification of gB viral sequences using Taqman real time PCR. Each bar represents one mouse. The lymph node is the periaortic lymph node. * is not quantifiable (1-15 copies; NQ). On day 24, there was a third mouse that only had NQ in prostate and periaortic lymph node (not shown in figure). For the second day 24 mouse, no DNA was obtained from the lung, including GAPDH.

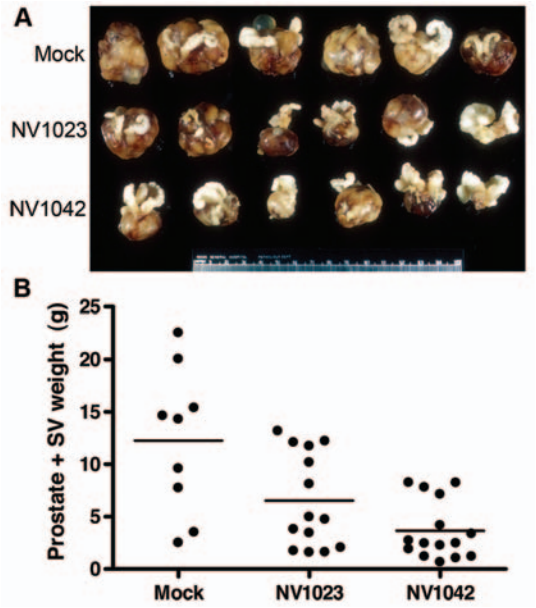
Varghese Figure 1



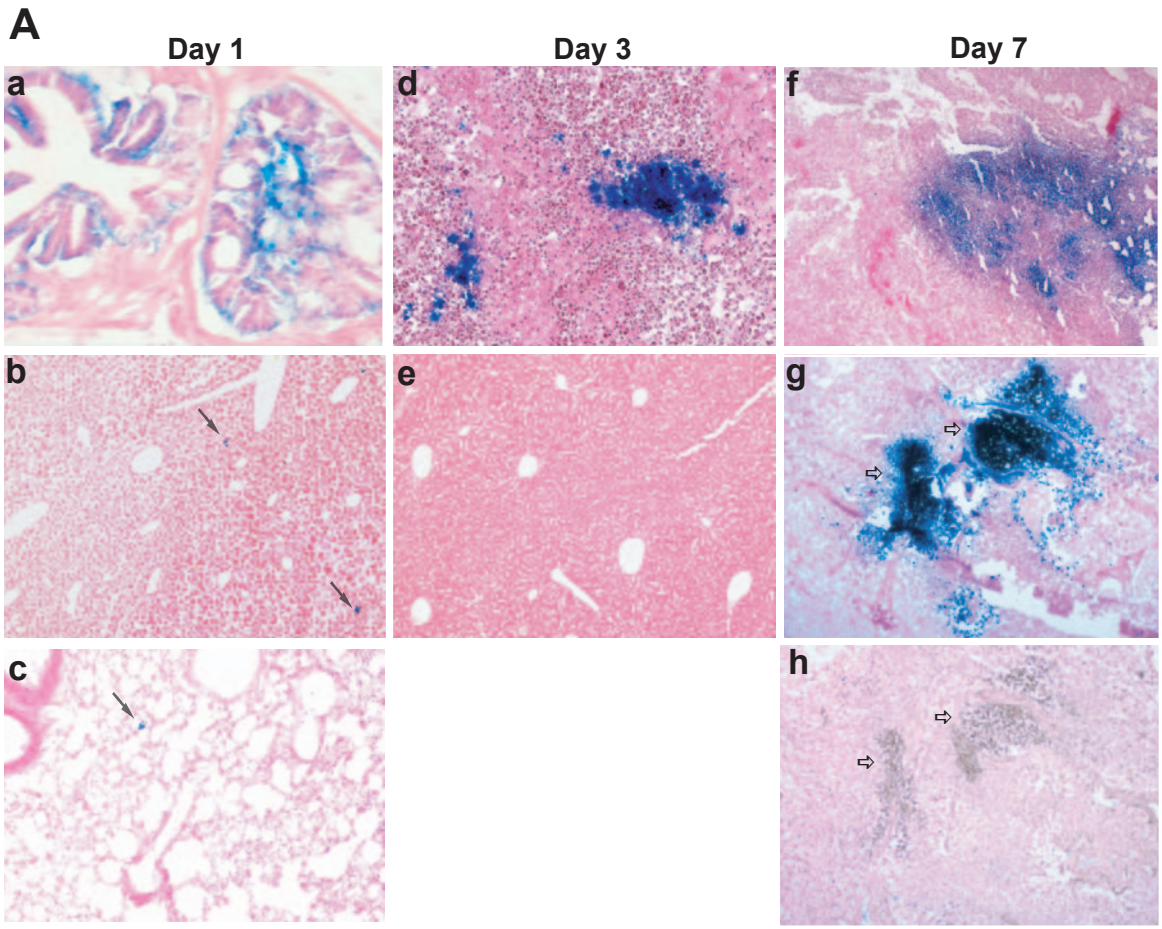
Varghese Figure 2

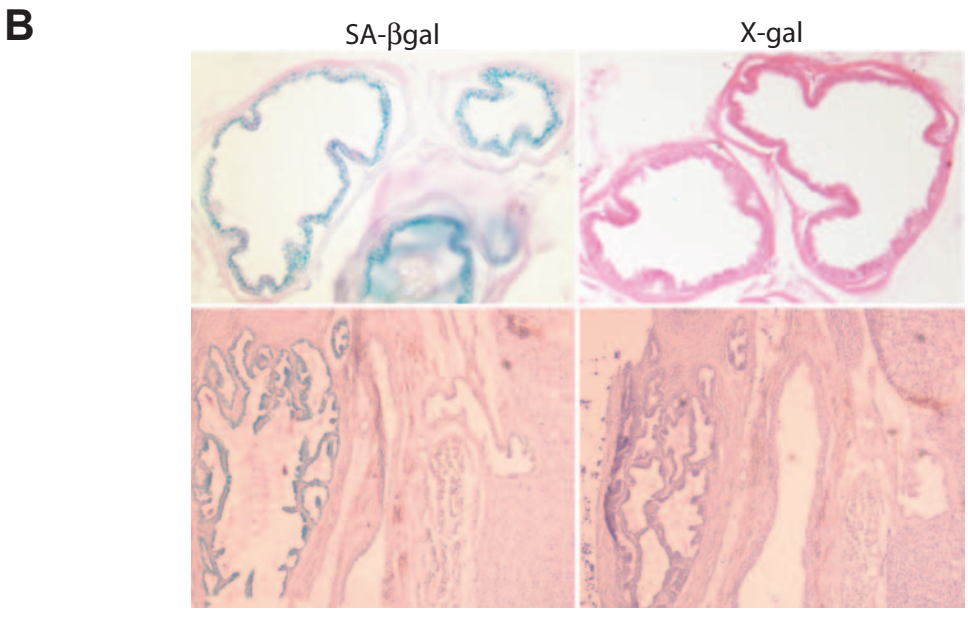


Varghese Figure 3



Varghese Figure 4





Varghese Figure 5

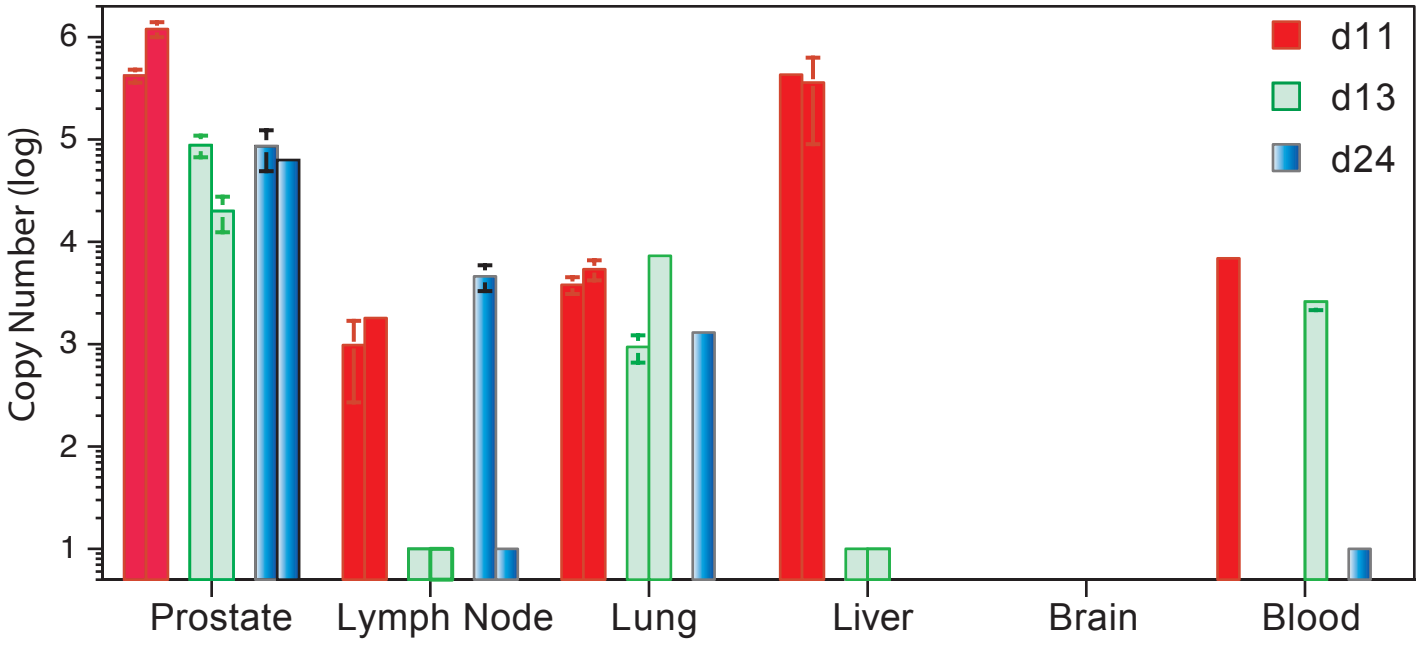


Table 1: Frequency of prostate lesions and metastatic cancer in TRAMP mice treated with oncolytic HSVs. $2x10^7$ pfu of NV1023 or NV1042 or virus buffer (Mock) was administered systemically on days 0, 3, 7, and 10 in TRAMP mice at either 12 or 18 weeks of age. Mice were sacrificed at 24 weeks and various tissues were processed for H&E staining and histologically graded. N values are shown in parenthesis under each group (s= number of mice sacrificed at 24 weeks of age, d= number of mice dead prior to 24 weeks of age). * $P < 0.05$ vs. Mock; [1] denotes combined total of the two types invasive carcinoma for statistical analysis.

Site/ Histology	Treatment at 12 Weeks of Age			Treatment at 18 Weeks of Age		
	Mock (n=7s+2d)	NV1023 (n=7s+2d)	NV1042 (n=8s)	Mock (n=9s+8d)	NV1023 (n=14s+3d)	NV1042 (n=15s+2d)
Prostate- Normal			1/8 (12.5%)			1/17 (6%)
Prostate- PIN	1/9 (11%)	3/9 (33%)	5/8 (63%)	1/17 (6%)	4/17 (24%)	6/17 (35%)
Prostate- Invasive Carcinoma: well differentiated adenocarcinoma					1/17 (6%)	1/17 (6%) *[1]
Prostate- Invasive Carcinoma: undifferentiated	8/9 (89%)	6/9 (67%)	2/8 (25%) *	16/17 (94%)	12/17 (71%)	9/17 (53%) *[1]
PA-LN Carcinoma	6/7 (86%)	1/7 (14%) *	2/8 (25%) *	8/9 (89%)	6/14 (43%) *	5/15 (33%) *
Lung Carcinoma	4/7 (57%)	1/7 (14%)	1/8 (12.5%)	5/9 (56%)	5/14 (36%)	1/14 (7%) *